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(54) Title: SUBSTRATE DETECTION ASSAY

(57) Abstract: Methods and compositions for evaluating nicotinamide-releasing activities are provided herein.

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SUBSTRATE DETECTION ASSAY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of U.S.S.N. 60/440,723, filed January 16, 2003, the contents of which are hereby incorporated by reference in its entirety.

BACKGROUND

The pyridine nucleotides NAD^+ and NADH are found in all living cells and serve as critical activating factors for numerous enzymatic processes. NAD^+ consists of an adenosine monophosphate (AMP) and a nicotinamide ring. The nicotinamide ring can accept two electrons and a proton to form NADH. This reduced form readily transfers a hydride ion due to reduced resonance stability.

NADH and NAD^+ play a central role in oxidative catabolism. They also have important non-redox activities as cellular effectors and metabolic regulators. Central to the many diverse biological activities of NAD^+ is the enzyme-catalyzed cleavage of the nicotinamide-ribosyl bond of NAD^+ and the attendant transfer of the ADP-ribosyl moiety to acceptors and release of nicotinamide. NAD-dependent enzymes include deacetylases, DNA ligases, aldehyde dehydrogenases, and toxins associated with cholera, diphtheria, pertussis. Other NAD-dependent activities include the reversible ADP-ribosylation-mediated biological regulatory systems; the synthesis of poly(ADP-ribose) in response to DNA damage or cellular division; and the synthesis of cyclic ADP-ribose as part of an independent, calcium-mediated regulatory system (Oppenheimer, Mol Cell Biochem 1994 Sep;138(1-2):245-51).

SUMMARY

In one aspect, the invention features a method of evaluating a sample. The method includes: providing a sample (e.g., including a known or unknown activity), and a donor substrate that includes (i) a nicotinamide moiety (e.g., NAD), (b) maintaining the sample under preselected conditions; (c) contacting the sample to a matrix that preferentially interacts with the donor substrate relative to nicotinamide; and (d) evaluating (i') components

of the contacted sample that do not interact with the matrix or (ii') components of the contacted sample that do interact with the matrix.

In one embodiment, the sample further includes: (ii) a nicotinamide-releasing activity; and optionally (iii) a test compound. The method can be used to evaluate one or more test compounds.

The evaluating can include determining a parameter characteristic of components of the contacted sample that do not interact with the matrix. For example, the parameter is a function of nicotinamide concentration (e.g., directly proportional to). The method can further include comparing the parameter to a reference value, e.g., a corresponding value for a control sample evaluated by the method. A control sample can be a sample lacking the activity, a sample having a known activity, a sample lacking a donor substrate, a sample which is not subjected to the contacting step of the method, a sample which is not contacted to the matrix, etc. The control sample is otherwise identical to the sample evaluated by the method and/or is subjected to a method which is otherwise identical.

In one embodiment, the contacting includes separating components of the contact sample that interact with the matrix from the matrix, thereby separating the donor substrate from the sample.

In one embodiment, the matrix covalently bonds to the donor substrate.

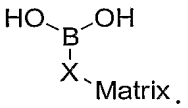
In one embodiment, the donor substrate is labelled. The detecting can include detecting the label. A label can be judiciously located, e.g., depending on whether molecules that interact with the matrix or molecules that do not interact with the matrix are evaluated. For example, the nicotinamide moiety portion of the donor substrate is labeled, or non-nicotinamide moiety portion is labeled. In one embodiment, the label is radioactive. In another embodiment, the label is not radioactive. For example, the label is fluorescent.

In one embodiment, the evaluating includes an enzyme-based assay. (e.g., a deamidase such as nicotinamide deamidase or a transferase such as nicotinamide N-methyl transferase assay).

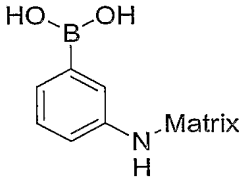
In one embodiment, the evaluating includes spectroscopic detection. In one embodiment, the separating does not require high-pressure liquid chromatography. For example, the separating can include one or more of: vacuum, gravity, or centrifugation. The

method can include using a container that includes a plurality of samples, e.g., at least 6, 10, 32, 64, or 100, or 300 samples.

In one embodiment, the matrix selectively interacts with (e.g., binds or couples to) compounds having 1,2-diols. For example, the matrix can include a boronate group attached thereto. The boronate group can be attached to the resin through a variety of linker moieties X as shown below.



X can include an aromatic moiety such as the aniline moiety below.



Additionally, X can include an alkyl moiety, an alkenyl moiety, an alkynyl moiety, a heteroaryl moiety, a cyclyl moiety, a heterocycly moiety, etc. In instances where the linker moiety is attached to the matrix through an amide bond, the linker moiety generally includes a nitrogen containing moiety (e.g., an amine containing moiety such as an analine) or a carbonyl moiety. In instance where the linker moiety is attached to the matrix through an ester bond, the linker moiety generally includes an oxygen containing moiety (e.g., a hydroxy containing moiety such as a phenol) or a carbonyl moiety. While amide and ester attachments are described herein, other means of attachment of the linker moiety to the matrix are also envisioned.

In one embodiment, the donor substrate is NAD, NADH, NADP, or NADPH, derivatives thereof, or a cofactor that can bind to a SIRT protein or an ADP ribosylase.

In one embodiment, the sample further includes an acetylated polypeptide, e.g., an acetylated peptide with fewer than 32, 20, or 15 amino acids. In another example, the acetylated polypeptide includes a SIRT polypeptide substrate. For example, the acetylated polypeptide includes 3, 4, 5, 11, 20, 40, or more amino acids (e.g., all amino acids) from p53, a histone (e.g., H3, H4, H2A, or H2B), a cytochrome, a cytoskeletal protein, a mitochondrial protein, a protein that mediates apoptosis, that regulates cell proliferation, or that regulates senescence.

The acetylated polypeptide can also have fewer than 20, 18, 15, 12, or 10 amino acids. Exemplary peptide substrates include peptides that have at one or more lysine (K) residues, *e.g.*, K370, K371, K372, K381, and/or K382 of human p53, or lysine positions in a non-human p53. In one embodiment, the peptide is residues 379-382 of p53 (Arg-His-Lys-Lys(Ac)). Another exemplary peptide substrate includes the acetylated N-terminal tail of a histone, e.g., H3 or H4. For example, the substrate can be the following tail of histone H4 (12-16, Lys-Gly-Gly-Ala-Lys(Ac))

Exemplary peptides include between 3 and 20, 3 and 12, 4 and 12, or 5 and 8 amino acids. Exemplary peptides can include exactly one lysine that is acetylated and/or exactly one lysine total.

The nicotinamide-releasing activity can be associated with a deacetylase activity (e.g. ability to catalyze deacetylation of an acetylated polypeptide, e.g., an acetylated lysine of a polypeptide), e.g., a deacetylase activity is associated with a histone deacetylase activity, a SIRT activity, or an activity that can deacetylate p53, a histone (e.g., H3, H4, H2A, or H2B), a cytochrome, a cytoskeletal protein, a mitochondrial protein, a protein that mediates apoptosis, that regulates cell proliferation, or that regulates senescence.

In one embodiment, the nicotinamide-releasing activity is a NAD hydrolase activity.

In one embodiment, a plurality of samples is provided and wherein a plurality of test compounds are evaluated by the method. The compounds can be compounds from a library, e.g., a library of test compounds described herein.

In one embodiment, the reaction mixture further includes an acceptor substrate, wherein the nicotinamide-releasing activity causes the acceptor substrate to become ADP-ribosylated. For example, the acceptor substrate is acetylated.

In one embodiment the test compound is stilbene or a derivative thereof, chalcone or a derivative thereof, or flavone a derivative thereof. Examples of stilbene derivatives include *trans*-stilbene, and hydroxy containing-*trans*-stilbene derivatives such as hydroxy-*trans*-stilbene, dihydroxy-*trans*-stilbene, trihydroxy-*trans*-stilbene (e.g., 3,5,4'- trihydroxy-*trans*-stilbene), tetrahydroxy-*trans*-stilbene (e.g., 3,5,3',4'- tetrahydroxy-*trans*-stilbene), etc. Examples of chalcone derivatives hydroxy containing chalcone derivatives such as hydroxychalcone, dihydroxychalcone, trihydroxychalcone (e.g., 4,2',4'- trihydroxychalcone), tetrahydroxychalcone (3,4,2'4'- tetrahydroxychalcone), etc. Examples of flavone derivatives

include hydroxy containing flavones such as hydroxyflavone, dihydroxyflavone, trihydroxyflavone, tetrahydroxyflavone (3,7,3',4'- tetrahydroxyflavone), pentahydroxyflavone (3,5,7,3',4'-pentahydroxyflavone) etc. While hydroxy containing derivatives of stilbene, chalcone and flavone have been described, other substituents are also envisioned, including but not limited to halo, alkyl, alkenyl, and alkoxy.

The method can further include formulating the test compound as a pharmaceutical. The method can further include administering the test compound or a formulation thereof to a subject, e.g., a mammalian subject, e.g., a mouse, or a human, e.g., a diseased subject, an adult subject, or another subject described herein. The method can further include contacting the test compound to a cell, e.g., a mammalian cell.

In one embodiment, the nicotinamide releasing activity can include a purified protein, e.g., a recombinant protein. In one embodiment, the recombinant protein is tagged.

In one embodiment, the purified protein is a mutant protein (e.g., a protein that includes a polypeptide having an amino acid with one or more insertions, deletions, or substitutions relative to another sequence, e.g., a naturally-occurring sequence or other reference sequence). A mutant protein can be made by mutagenesis of a template nucleic acid that encodes a reference protein. The invention includes evaluating one or more mutant proteins using a method described herein. The mutant protein can include a fragment, e.g., at least 20, 40, 50, or 80 amino acids of another protein, e.g., a functional fragment.

In one embodiment, the sample is a patient sample or a fraction thereof (e.g., a membrane fraction of a cellular extract, a protein-clarified extract, a nuclear extract, or a cytoplasmic extract). For example, a sample from a polypeptide can be contacted with an antibody that is specific for a polypeptide that has nicotinamide releasing activity, e.g., a SIRT protein, to provide a fraction enriched (or at least 10% purified for the SIRT protein).

In one embodiment, the nicotinamide releasing activity is immobilized during the maintaining, e.g., immobilized to a planar substrate, a bead, a reaction vessel, etc.

Exemplary preselected conditions include a preselected temperature or temperature range, e.g., between 0-50, 4-42, 10-40, 10-20, 20-30, 25-40, 30-40, 30-35, 35-40, or 36-39°C. Exemplary conditions can include a preselected pH, e.g., between 5-9, 6-8, 5-7, or 7-9.

In a related aspect, the invention features a method of evaluating a sample. The method includes: providing a sample (e.g., including a known or unknown activity), e.g., the

sample including (i) a compound that includes a ribose containing moiety, (b) maintaining the sample, e.g., under preselected conditions; (c) contacting the sample to a matrix that preferentially interacts with a reactant relative to a reaction product, or that preferentially interacts with a reaction product relative to a reactant; and (d) evaluating components of the contacted sample that do not interact with the matrix, or components of the sample that do interact with the matrix.

In the case of a matrix that can bind to other ribose containing moieties, the matrix can be used to evaluate a reaction (e.g., an enzymatic reaction) in which the compound that includes a ribose containing moiety is modified. For example, the method can be used evaluate a reaction (or activity of a reaction component, e.g., an enzyme) for ability to modify the compound. The modification may cause a moiety (e.g., a labeled moiety) to be covalently linked to the compound, or may cause a moiety (e.g., a labeled moiety) to be released from the compound (e.g., by breaking a covalent bond).

The compound may include a ribose moiety bound to a nitrogen containing heteroaryl moiety. The nitrogen containing heteroaryl moiety can be substituted, for example with an amide moiety, a hydroxy moiety, a cyano moiety, an ester moiety, a nitro moiety, etc. In some instances, the nitrogen containing heteroaryl moiety is substituted with multiple substituents. In some instances the nitrogen contining heteroaryl moiety is labeled, for example using a radioisotope or a fluorescent probe.

Examples of nitrogen containing heteroaryl moieties include, but are not limited to pyridine, pyrimidine, pyrazine, pyridazine, pyrrole, pyrrolopyrimidine, etc.

The method can include other features, e.g., other features described herein.

In another aspect, the invention features a method that includes: (a) providing a sample; (b) contacting the sample with a NAD-interacting matrix, wherein the matrix does not interact with nicotinamide, under conditions that allow the NAD to bind (e.g. couple to) the matrix; and (c) detecting nicotinamide after the contacting. The method can include other features described herein.

In one embodiment, the sample is obtained by lysing a cell, or combining components, e.g., purified components (one or more of the components can be at least 10, 20, 50, 70, 80, 90, 95, or 99% pure). Exemplary samples include extracts, e.g., nuclear extracts,

cytoplasmic extracts, clarified extracts, lipid free extracts, protein only fraction, protein size fractionated fractions, chromatographic separation fractions, and so forth.

In another aspect, the invention features a method that includes: (a) providing a sample; (b) contacting the sample to a nicotinamide modifying activity; and (c) detecting a product produced by a reaction catalyzed by the nicotinamide modifying activity. The sample can include, for example, (i) a sample having nicotinamide-releasing activity; (ii) a donor substrate, wherein the donor substrate includes a nicotinamide moiety. In one embodiment, the sample further includes (iii) a test compound. Prior to the contacting, the method can further include: maintaining the sample under preselected conditions.

In one embodiment, the detecting includes detecting a fluorescence or colorimetric product, e.g., using spectroscopy.

In one embodiment, the nicotinamide-modifying activity is nicotinamide deamidase activity. In one embodiment, the product is ammonia, and the ammonia is detected, e.g., by contacting the reaction mixture with o-phthaldialdehyde (OPA) (e.g., and also sodium sulfite, and sodium borate); and evaluating an optical property of the reaction mixture, e.g., fluorescence, thereby detecting levels of ammonia released.

In one embodiment, a plurality of samples is provided and a plurality of test compounds are evaluated.

In one embodiment, the nicotinamide-modifying enzyme is nicotinamide N-methyl transferase, and wherein the modified nicotinamide is detected.

In one embodiment, the contacting step further includes contacting the sample with acetophenone/KOH and formic acid, and heating the sample.

In one embodiment, the detecting includes detecting fluorescence (OD).

The method can further include determining a parameter characteristic of the detected sample. For example, the parameter is a function of nicotinamide concentration (e.g., directly proportional to nicotinamide concentration). The method can further include comparing or correlating the parameter to a reference value, e.g., a corresponding value for a control sample evaluated by the method. Exemplary control samples include a sample that is not contacted to the nicotinamide-modifying activity, a sample contacted to a known level of nicotinamide-modifying activity, a sample lacking a donor substrate, a sample lacking a test

compound, etc. The control sample can be otherwise identical to the sample evaluated by the method and/or is subjected to a method which is otherwise identical.

The method can further include other features described herein.

In one aspect, the invention features a method of evaluating a nicotinamide-releasing activity. The method includes: (a) providing a sample including (i) nicotinamide-releasing activity, and (ii) a donor substrate, wherein the donor substrate includes a nicotinamide moiety; (b) contacting the reaction mixture with nicotinamide-modifying activity under conditions that allow the enzyme to react with nicotinamide; and (c) evaluating one or more of: a nicotinamide that has been modified by the enzyme, or a product of the nicotinamide-modifying activity. The method can include other features described herein. A related method can be used to evaluate a nicotinamide binding activity, a reaction that produces nicotinamide as a product, or a reaction that uses nicotinamide as a substrate.

In still another aspect, the invention includes a method of detecting a deactylase activity, e.g., ahistone deacetylase activity. The method includes: providing a sample having deacetylase activity (e.g., a histone deacteylase) or a sirtuin protein; maintaining the sample under preselected conditions; and detecting or evaluating nicotinamide. The sample can include an acetylated substrate of the histone deacteylase or the sirtuin protein. In one embodiment, the sample further includes a donor substrate, wherein the donor substrate includes a nicotinamide moiety.

In one embodiment, the method can further include, prior to the detecting, separating the nicotinamide from the donor substrate by binding the sample to a matrix that selectively interacts with the donor substrate but not the nicotinamide, wherein the binding is performed under conditions that allow the unreacted donor substrate to bind the matrix. In another embodiment, the method further includes contacting the sample with a nicotinamide-modifying enzyme under conditions that allow the nicotinamide-modifying enzyme to react with the nicotinamide; and detecting a product of a reaction catalyzed by the nicotinamide modifying enzyme. The method can include other features described herein.

In another aspect, the invention features a method of purifying a nicotinamide releasing activity. The method includes assaying one or more fraction from a separation procedure using a method described herein. The method can be used to purify a nicotinamide releasing activity, e.g., a sirtuin protein.

In another aspect, the invention features a kit including: a control enzyme having nicotinamide-releasing activity; a nicotinamide-modifying enzyme; and NAD.

The kit can further include: instructions for detecting one of: modified nicotinamide, or a byproduct of an activity of the nicotinamide-modifying enzyme, e.g., using a method described herein. In another aspect, the invention features a kit including: a nicotinamide-binding matrix; NAD, an acetylated acceptor peptide; and optionally a control enzyme and instructions for use in detecting nicotinamide-releasing activity.

In one aspect, this invention provides methods to evaluate the activity of nicotinamide-releasing enzymes, such as the SIRT class of deacetylases and the NAD hydrolases. SIRT enzymes are implicated in aging and age-related diseases such as cancer. The methods enable, for example, careful kinetic analysis and high-throughput testing of compounds as potential inhibitors and activators.

In one embodiment, the assay is implemented for a plurality of samples (which may include different components or different conditions, or which may include a common set of components or conditions, and one variable element (e.g., a test compound). The method can be implemented using a single plate and/or homogeneous fluorescent detection that is highly sensitive and miniaturizable. In one embodiment, the method does not use radioactive materials, thereby avoiding associated handling and disposal issues.

The invention also features the of boronate resins in a multi-sample container, e.g., a multi-well plate such as a microplate, (e.g., a Multiscreen plate from Millipore Corp). Exemplary boronate resins are commercially available from Pierce (*www.piercenet.com*) and Bio-Rad (*www.biorad.com*).

In one embodiment, a method described herein is performed without the use of column separation, e.g., without chromatography and/or without solvent extraction. The method can include elution of bound radiolabeled product before scintillation counting.

The invention also features a method for evaluating an enzyme that includes contacting a reaction mixture (or fraction thereof) to a boronate resin to separate nicotinamide from NAD as the basis for an enzyme assay. In another aspect, the invention features a method for evaluating an enzyme that includes providing nicotinamide-modifying activity to the reaction mixture (or a fraction thereof) and evaluating a product of the nicotinamide-modifying activity. In another aspect, the invention features direct fluorometric

detection of ammonia in an enzyme assay, e.g., of an enzyme that produces a product which can be used as a substrate for ammonia detection.

In some implementations the assays of nicotinamide release allow miniaturization, e.g., to 96- and 384-well microplate format, protein array, protein chip, or micro-chip formats. In many implementations, the methods are non-radioactive, homogeneous methods. The fluorescent readouts are highly sensitive and amenable to miniaturization and avoid the need to handle radioactive materials.

The nicotinamide detection method can also be used to avoid evaluating depletion of substrate, e.g., NAD depletion.

A streamlined assay for nicotinamide releasing enzymes (e.g., SIRT enzymes) can be used, for example, for enzymatic characterization and for profiling of test compounds, e.g., inhibitors, e.g., for potency and selectivity. The assays can be implemented for high-throughput screening.

A sample can be a biological sample, e.g., from an organism (e.g., blood, biopsy, cells) or from cultured cells, or a protein sample (e.g., purified protein), or a cellular extract.

"Nicotinamide-releasing activity" refers to an activity that produces nicotinamide (or a molecule that includes nicotinamide-containing moiety) from a precursor compound (e.g., NAD). Generally the activity is an enzymatic activity. For example, the nicotinamide-releasing activity can be an enzyme, e.g., an enzyme that can interact with NAD, NADP, NADH, a ribonucleoside, a ribonucleotides, or nicotinamide. "Nicotinamide-modifying activity" refers to an activity that causes modification of nicotinamide molecules or of molecules that include a nicotinamide moiety.

The term "matrix" refers to any insoluble support, e.g., a particle (e.g., a magnetic particle, porous particle), a bead, a planar surface, a resin, a gel (e.g., agarose, or polyacrylamide gel), all or part of a reaction vessel such as a multi-container sample carrier (e.g., a microtitre plate), tube, column, spin-cup, disposable pipet tip, ring, disc (e.g., paper disc), membrane. For example, the templates can be attached to a surface within one or more microtitre wells (e.g., in a variety of formats, including single, strips, 96-well, 384-well, robotically manipulated single or multiple plates).

The term "polypeptide" refers to a polymer of three or more amino acids linked by a peptide bond. The polypeptide may include one or more unnatural amino acids. Typically,

the polypeptide includes only natural amino acids. The term "peptide" refers to a polypeptide that is between three and thirty-two amino acids in length. A "protein" can include one or more polypeptide chains. Accordingly, the term "protein" encompasses polypeptides and peptides. A protein or polypeptide can also include one or more modifications, e.g., an acetylation, glycosylation, amidation, phosphorylation, and so forth.

The term "isolated nucleic acid molecule" or "purified nucleic acid molecule" includes nucleic acid molecules that are separated from other nucleic acid molecules present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. In some embodiments, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and/or 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of 5' and/or 3' nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Examples of flanking sequences include adjacent genes, transposons, and regulatory sequences. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, of culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in Nature. For example a naturally occurring nucleic acid molecule can encode a natural protein.

As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include at least an open reading frame encoding a protein, a protein subunit, derivative, or functional domain thereof. The gene can optionally further include non-coding sequences, e.g., regulatory sequences and introns.

An "isolated" or "purified" polypeptide or protein is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. "Substantially free" means that the protein of interest in the

preparation is at least 10% pure. In an embodiment, the preparation of the protein has less than about 30%, 20%, 10% and more preferably 5% (by dry weight), of a contaminating component (e.g., a protein not of interest, chemical precursors, and so forth). When the protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation. The invention includes isolated or purified preparations of at least 0.01, 0.1, 1.0, and 10 milligrams in dry weight.

In one embodiment, the methods of the invention can be used to evaluate "SIRT proteins" and "SIRT polypeptides" are used interchangeably herein and refer to members of the Silent Information Regulator (SIR) family of genes. In particular, the term "SIRT1 protein" or "SIRT1 polypeptide" refers to a polypeptide that is at least 25% identical to a conserved SIRT catalytic domain, amino acid residues 258 to 451 of SEQ ID NO:1 (shown in Table 1, below). Likewise for other SIRT proteins known or described herein (see, e.g., Table 2).

Table 1. Human SIRT1 Amino Acid Sequence

GenBank® GI: 9884660, Acc:CAC04174.1| bA57G10.4 (SIRT1, Sir2-like proteins (siruitins) type 1) (Homo sapiens)

MIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPKRKKRKDINTIEDAVKLLQECKKIIV
LTGAGVSVSCGIPDFRSRDGIYARLAVDFPDLPDPQAMFDIEYFRKDPRPFFKFAKEIYPGQFQ
PSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDCEAVRGDI
FNQVVPRCPRCPADEPLAIMKPEIVFFGENLPEQFHRAMKYDKDEVDLLIVIGSSLKVRPVALIP
SSIPHEVPQILINREPLPHLHFDVELLGDCDVIINELCHRLGGEYAKLCCNPVKLSEITEKPPRT
QKELAYLSELPPTPLHVSEDSSSPERTSPPDSSVIVTLLDQAAKSNDDLDVSESKGCMEEKPQE
VQTSRNVESIAEQMENPDLKNVGSSTGEKNERTSVAGTVRKCWPNRVAKEQISRRLDGNQY
LFLPPNRYIFHGAEVYSDSEDDVLSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFYNGLEDEP
DVPERAGGAGFGTDGDDQEAINEAISVKQEVTDMNYPSNKS (SEQ ID NO:1)

Other exemplary SIRT proteins include SIRT2 and SIRT3.

In some embodiments, a SIRT polypeptide can be at least 30, 40, 50, 60, 70, 80, 85, 90, 95, 99% homologous to a reference sequence. In other embodiments, the SIRT1 polypeptide can be a fragment, e.g., a fragment of SIRT1 capable of one or more of: deacetylating a substrate in the presence of NAD and/or a NAD analog and capable of

binding a target protein, e.g., a transcription factor, e.g., p53 or a transcription factor other than p53. Such functions can be evaluated, e.g., by the methods described herein. In other embodiments, the SIRT polypeptide can be a "full length" SIRT polypeptide. The term "full length" as used herein refers to a polypeptide that has at least the length of a naturally-occurring SIRT polypeptide (or other protein described herein). A "full length" SIRT polypeptide or a fragment thereof can also include other sequences, e.g., a purification tag., or other attached compounds, e.g., an attached fluorophore, or cofactor. The term "SIRT polypeptides" can also include sequences or variants that include one or more substitutions, e.g., between one and ten substitutions, with respect to a naturally occurring Sir2 family member. In preferred embodiments, a human SIRT polypeptide can vary from SEQ ID NO:1 by at least 1, 2, 3, 4, 5, 10, 15, but preferably not more than 20 to 50 amino acid residues, e.g., it can vary by at least 1, 2, 3, 4, 5, 10, 15 substitutions, e.g., conservative substitutions. In other embodiments, the SIRT1 polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid encoding the amino acid sequence of SEQ ID NO:1, e.g., at least amino acid residues 258 to 451 of SEQ ID NO:1. The term "SIRT polypeptide" is also includes homologs of human SIRT proteins from other species including the murine homolog of SIRT1, also referred to as "Sir2α". A "SIRT1 activity" refers to one or more activity of SIRT1, *e.g.*, deacetylation of transcription factors such as p53 or histone proteins, (e.g., in the presence of a cofactor such as NAD and/or an NAD analog) and binding of a target protein, e.g., a transcription factor, e.g., p53 or a transcription factor other than p53.

A variety of methods can be used to identify a SIRT family member. For example, a known amino acid sequence of a known SIRT protein can be searched against the GenBank® sequence databases (National Center for Biotechnology Information, National Institutes of Health, Bethesda MD), e.g., using BLAST; against Pfam database of HMMs (Hidden Markov Models) (using default parameters for Pfam searching; against the SMART database; or against the ProDom database. For example, the hmmsf program, which is available as part of the HMMER package of search programs, is a family specific default program for MILPAT0063 and a score of 15 is the default threshold score for determining a hit. Alternatively, the threshold score for determining a hit can be lowered (e.g., to 8 bits). A description of the Pfam database can be found in Sonhammer *et al.* (1997) *Proteins*

28(3):405-420 and a detailed description of HMMs can be found, for example, in Gribskov *et al.* (1990) *Meth. Enzymol.* 183:146-159; Gribskov *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4355-4358; Krogh *et al.* (1994) *J. Mol. Biol.* 235:1501-1531; and Stultz *et al.* (1993) *Protein Sci.* 2:305-314. The SMART database (Simple Modular Architecture Research Tool, EMBL, Heidelberg, DE) of HMMs as described in Schultz *et al.* (1998), *Proc. Natl. Acad. Sci. USA* 95:5857 and Schultz *et al.* (200) *Nucl. Acids Res* 28:231. The SMART database contains domains identified by profiling with the hidden Markov models of the HMMer2 search program (R. Durbin *et al.* (1998) *Biological sequence analysis: probabilistic models of proteins and nucleic acids.* Cambridge University Press). The database also is annotated and monitored. The ProDom protein domain database consists of an automatic compilation of homologous domains. (Corpet *et al.* (1999), *Nucl. Acids Res.* 27:263-267) Current versions of ProDom are built using recursive PSI-BLAST searches (Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402; Gouzy *et al.* (1999) *Computers and Chemistry* 23:333-340.) of the SWISS-PROT 38 and TREMBL protein databases. The database automatically generates a consensus sequence for each domain.

Calculations of homology or sequence identity between sequences (the terms are used interchangeably herein) are performed as follows.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology").

The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. The comparison is generally done using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

The nucleic acid and protein sequences described herein can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

Some polypeptides of the present invention can have an amino acid sequence substantially identical to an amino acid sequence described herein. In the context of an amino acid sequence, the term "substantially identical" is used herein to refer to a first amino acid that contains a sufficient or minimum number of amino acid residues that are i) identical to, or ii) conservative substitutions of aligned amino acid residues in a second amino acid sequence such that the first and second amino acid sequences can have a common structural domain and/or common functional activity. Methods of the invention can include use of a polypeptide that includes an amino acid sequence that contains a structural domain having at least about 60%, or 65% identity, likely 75% identity, more likely 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% identity to a domain of a polypeptide described herein.

In the context of nucleotide sequence, the term "substantially identical" is used herein to refer to a first nucleic acid sequence that contains a sufficient or minimum number of

nucleotides that are identical to aligned nucleotides in a second nucleic acid sequence such that the first and second nucleotide sequences encode a polypeptide having common functional activity, or encode a common structural polypeptide domain or a common functional polypeptide activity. Methods of the invention can include use of a nucleic acid that includes a region at least about 60%, or 65% identity, likely 75% identity, more likely 85%, 90%. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to a nucleic acid sequence described herein, or use of a protein encoded by such nucleic acid.

A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of protein without abolishing or substantially altering activity, e.g., the activity is at least 20%, 40%, 60%, 70% or 80% of wild-type. An "essential" amino acid residue is a residue that, when altered from the wild-type sequence results in abolishing activity such that less than 20% of the wild-type activity is present. Conserved amino acid residues are frequently predicted to be particularly unamenable to alteration.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

As used herein, a "biologically active portion" or a "functional domain" of a protein includes a fragment of a protein of interest which participates in an interaction, e.g., an intramolecular or an inter-molecular interaction, e.g., a binding or catalytic interaction. An

inter-molecular interaction can be a specific binding interaction or an enzymatic interaction (e.g., the interaction can be transient and a covalent bond is formed or broken). An inter-molecular interaction can be between the protein and another protein, between the protein and another compound, or between a first molecule and a second molecule of the protein (e.g., a dimerization interaction). Biologically active portions/functional domains of a protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the protein which include fewer amino acids than the full length, natural protein, and exhibit at least one activity of the natural protein. Biological active portions/functional domains can be identified by a variety of techniques including truncation analysis, site-directed mutagenesis, and proteolysis. Mutants or proteolytic fragments can be assayed for activity by an appropriate biochemical or biological (e.g., genetic) assay. In some embodiments, a functional domain is independently folded. Typically, biologically active portions comprise a domain or motif with at least one activity of the protein, e.g., a nicotinamide-releasing activity or an NAD-dependent activity.

"NAD" refers to nicotinamide adenine dinucleotide. An "NAD analog" as used herein refers to a compound (*e.g.*, a synthetic or naturally occurring organic molecule) which possesses structural similarity to component groups of NAD (*e.g.*, adenine, ribose and phosphate groups) or functional similarity. For example, an NAD analog can be 3-aminobenzamide or 1,3-dihydroisoquinoline (H. Vaziri *et al.*, EMBO J. 16:6018-6033 (1997)).

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims. All cited patents, patent applications (published and unpublished), and references (including references to public sequence database entries) are incorporated by reference in their entireties for all purposes. U.S.S.N. 60/440,723, filed January 16, 2003, U.S.S.N. 10/191,121 (published as US20040005574-A1), and U.S.S.N. 09/461,580 (published as US20030207325-A1) are incorporated by reference in their entireties for all purposes.

DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic depiction of the chemical structures of ^{14}C-NAD, ^{14}C-NAD bound to a boronate-based affinity resin, and ^{14}C-nicotinamide in steps of an exemplary nicotinamide release assay in which nicotinamide is quantitated after flowing through the affinity resin.

FIG. 2 is a schematic diagram of an exemplary nicotinamide release assay in which ^{14}C-NAD and an acetylated peptide are incubated with a sample such that ^{14}C-nicotinamide, peptide, and O-actetyl-ADP-ribose are generated by an activity in the sample. The supernatant of the sample is diluted with ammonium acetate and transferred to a filter plate in which it is mixed with a boronate resin. The supernatant is then filtered, and ^{14}C-nicotinamide in the filtrate is quantitated.

FIG. 3 is a schematic depiction of the chemical structures of ^{14}C-NAD, ^{14}C-NAD bound to a boronate-based affinity resin, and ^{14}C-nicotinamide in steps of an exemplary nicotinamide release assay in which nicotinamide is quantitated after separation from unreacted ^{14}C-NAD which is bound to the affinity resin.

FIG. 4 is a schematic diagram of an exemplary nicotinamide release assay in which ^{14}C-NAD and an acetylated peptide are incubated with a sample such that ^{14}C-nicotinamide, peptide, and O-actetyl-ADP-ribose are generated by an activity in the sample. The sample is mixed with a boronate resin and diluted with ammonium acetate. After the resin settles, the supernatant is removed and nicotinamide in the removed supernatant is quantitated.

FIG. 5 is a schematic depiction of the chemical structures of NAD, nicotinamide, and nicotinic acid in steps of an exemplary nicotinamide release assay. The ammonia generated by the activity of nicotinamide deamidase on nicotinamide is detected with a fluorometric assay after reaction with o-phthaldealdehyde (OPA), sodium sulfite, and sodium borate.

FIG. 6 is a schematic diagram of an exemplary nicotinamide release assay in which NAD and an acetylated peptide are incubated with a sample such that nicotinamide, peptide, and O-acetyl-ADP-ribose are generated. Next, the sample is incubated with nicotinamide deamidase. OPA is added and incubated with the sample, and fluorescence is detected.

FIG. 7 is a schematic depiction of the chemical structures of NAD, nicotinamide, N-methyl nicotinamide, and the product of N-methyl nicotinamide after reaction with acetophenone/KOH and formic acid.

FIG. 8 is a schematic diagram of an exemplary nicotinamide release assay in which NAD and acetylated peptide are incubated with a sample such that nicotinamide, peptide, and O-acetyl-ADP-ribose are generated. Next, the sample is incubated with nicotinamide N-methyl transferase (NNMT). Acetophenone/KOH and formic acid are added, and fluorescence in the reaction mixture is detected.

FIG. 9 is a graph depicting the results of an exemplary nicotinamide release assay in which samples containing ^{14}C-NAD or ^{14}C-nicotinamide were mixed with a boronate resin. The samples were filtered, and counts per minute (CPM) in the filtrate and total mixture were detected.

FIG. 10A and FIG. 10B are graphs depicting the results of an exemplary nicotinamide release assay in which ^{14}C-NAD and an acetylated peptide, p53-382, were incubated with human SIRT1. Cpm of ^{14}C-nicotinamide was determined after the reacted sample was filtered through a boronate resin. In FIG. 10A, the results are depicted as cpm versus the concentration of the substrate peptide. In FIG. 10B, the results are depicted as cpm versus the concentration of NAD.

DETAILED DESCRIPTION

The invention is based, *inter alia*, on novel evaluation techniques that can be used to evaluate nicotinamide and other related compounds. In some implementations, the assays can be used to detect release of nicotinamide, e.g., by an enzyme. The assays are useful for evaluating enzymes directly or indirectly, e.g., by detecting the release of nicotinamide. For example, the activity of NAD-dependent enzymes can be evaluated with these assays. Exemplary NAD-dependent enzymes include NAD-hydrolases, deacetylases, DNA ligases, aldehyde dehydrogenases, and toxins associated with cholera, diphtheria, pertussis.

The assays described here include, for example, assays in which a sample is contacted to a matrix that selectively binds a precursor of nicotinamide (e.g., NAD), and that does not bind nicotinamide, such that nicotinamide generated in a sample (e.g., by an enzymatic reaction) can be separated from the matrix. Other exemplary assays detect nicotinamide after treatment with a nicotinamide-modifying enzyme. Enzymes such as nicotinamide deamidase and nicotinamide N-methyl transferase react with nicotinamide and produce detectable compounds, or precursors of detectable compounds.

Nicotinamide-releasing enzymes

Exemplary nicotinamide-releasing enzymes include NAD-dependent histone deacetylases (which may have substrates other than histones), the SIRT class of deacetylases, as well as other nicotinamide-releasing enzymes. Nicotinamide-releasing enzymes can include NAD-dependent histone deacetylases (e.g., Sir2), NAD hydrolases, NAD glycohydrolases (Balducci and Micossi,
Mol Cell Biochem. 233(1-2):127-32, 2002), NAD(+) glycohydrolases (Tono-oka and Hatakeyama, Chem Pharm Bull.50(6):831-3, 2002), Poly(ADP-ribose) polymerase (Ha et al., Proc Natl Acad Sci U S A 99(1):245-50, 2002), and bacterial toxins (e.g., diphtheria toxin, Kahn and Bruice, J Am Chem Soc. 123(48):11960-9, 2001).

NAD-dependent histone deacetylases and substrates

NAD-dependent histone deacetylases can catalyze a NAD-nicotinamide exchange reaction that requires the presence of acetylated lysines such as those found in the N termini of histones. These enzymes are distinguished from other classes of deacetylases in that the histone deacetylation reaction absolutely requires NAD. The enzymes are active on histone substrates that have been acetylated by both chromatin assembly-linked and transcription-related acetyltransferases. These enzymes may also ADP-ribosylate histones.

Examples of NAD-dependent histone deacetylases include Sir2 of *S. cerevisiae* (reviewed in Guarente, 2000; Shore, 2000) and Sir2 homologs (Imai *et al.*, 2000; Smith *et al.*, 2000). Deacetylation of acetyl-lysine by Sir2 is tightly coupled to NAD hydrolysis. It has been reported that this reaction produces nicotinamide and a novel acetyl-ADP ribose compound (1-O-acetyl-ADP-ribose) (Tanner *et al.*, 2000; Landry *et al.*, 2000b; Tanny and Moazed, 2001).

The novel assays described herein are exemplified by Assay A, Assay B, Assay C, and Assay D, described below. While the assays are depicted with SIRT as a nicotinamide-releasing enzyme, it is understood that other nicotinamide-releasing enzymes and may be evaluated in these assays.

Assay A: Filtration Assay of ^{14}C-nicotinamide Release

This method is based on the use of boronate-based affinity resin that selectively binds 1,2-diols. ^{14}C-labeled NAD and acetylated substrate are incubated with SIRT enzyme. Following the enzymatic reaction, the release of ^{14}C-nicotinamide from NAD may be quantified by filtration of the reaction mixture through boronate resin. The chemical structures of NAD and nicotinamide in the steps of the assay are depicted in FIG. 1. The resin selectively binds excess unreacted NAD while allowing nicotinamide to flow through unbound (FIG. 2).

B: Resin-binding Assay of ^{14}C-nicotinamide Release

This method is the same as Assay A except that the enzymatic reaction and the resin binding are performed in the same plate. Following the enzymatic reaction, a slurry of resin is added, mixed well, and allowed to settle. The chemical structures of NAD and nicotinamide in the steps of the assay are depicted in FIG. 3. The resin selectively binds excess unreacted NAD while allowing nicotinamide to remain in the supernatant (FIG. 4). A portion of the supernatant is transferred to a second plate for quantification of ^{14}C-nicotinamide release by scintillation counting.

C: Coupled Enzymatic Assay of Nicotinamide Release (ammonia detection)

This method is based on enzymatic release of ammonia from nicotinamide and subsequent fluorometric ammonia detection. NAD and acetylated substrate are incubated with SIRT enzyme. The nicotinamide released in the SIRT reaction is then converted to nicotinic acid and ammonia by addition of the enzyme nicotinamide deamidase. The chemical structures of NAD, nicotinamide, and nicotinic acid in steps of this assay are depicted in FIG. 5. Ammonia is then detected by fluorometric assay using *o*-phthaldealdehyde (OPA) (FIG. 6).

D: Coupled Enzymatic Assay of Nicotinamide Release (*N*-methyl nicotinamide detection)

This method is based on enzymatic conversion of nicotinamide to *N*-methyl nicotinamide followed by reaction of the latter with acetophenone to generate a fluorescent

product. The structures of NAD, nicotinamide, and products of reacted nicotinamide are shown in FIG.7. NAD and acetylated substrate (e.g., an acetylated peptide) are incubated with SIRT enzyme. The nicotinamide released in the SIRT reaction is then converted to *N*-methyl nicotinamide by addition of the enzyme nicotinamide *N*-methyl transferase (NNMT). Base-catalyzed reaction with acetophenone followed by acidification of the condensation product with formic acid results in ring closure to form a fluorescent product (FIG. 8).

Test Compounds and Libraries

A "compound" or "test compound" can be any chemical compound, for example, a macromolecule (e.g., a polypeptide, a protein complex, or a nucleic acid) or a small molecule (e.g., an amino acid, a nucleotide, an organic or inorganic compound). The test compound can have a formula weight of less than about 10,000 grams per mole, less than 5,000 grams per mole, less than 1,000 grams per mole, or less than about 500 grams per mole. The test compound can be naturally occurring (e.g., a herb or a nature product), synthetic, or both. Examples of macromolecules are proteins, protein complexes, and glycoproteins, nucleic acids, e.g., DNA, RNA and PNA (peptide nucleic acid). Examples of small molecules are peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds e.g., heteroorganic or organometallic compounds. A test compound can be the only substance assayed by the method described herein. Alternatively, a collection of test compounds can be assayed either consecutively or concurrently by the methods described herein.

Compounds that bind and/or modulate the activity of nicotinamide-releasing enzymes have been described. See, for example, Howitz, et. al. *Nature*, 425(6954): 191-6, 2003. Compounds that can be tested in the methods described herein include such compounds and derivatives thereof.

Exemplary test compounds include stilbenes and derivatives thereof, chalcones and derivatives thereof, and flavones and derivative thereof. Examples of stilbene derivatives include *trans*-stilbene, and hydroxy containing-*trans*-stilbene derivatives such as hydroxy-*trans*-stilbene, dihydroxy-*trans*-stilbene, trihydroxy-*trans*-stilbene (e.g., 3,5,4'- trihydroxy-*trans*-stilbene), tetrahydroxy-*trans*-stilbene (e.g., 3,5,3',4'- tetrahydroxy-*trans*-stilbene), etc.

Examples of chalcone derivatives hydroxy containing chalcone derivatives such as hydroxychalcone, dihydroxychalcone, trihydroxychalcone (e.g., 4,2',4'- trihydroxychalcone), tetrahydroxychalcone (3,4,2'4'- tetrahydroxychalcone), etc. Examples of flavone derivatives include hydroxy containing flavones such as hydroxyflavone, dihydroxyflavone, trihydroxyflavone, tetrahydroxyflavone (3,7,3',4'- tetrahydroxyflavone), pentahydroxyflavone (3,5,7,3',4'-pentahydroxyflavone) etc. While hydroxy containing derivatives of stilbene, chalcone and flavone have been described, other substituents are also envisioned, including but not limited to halo, alkyl, alkenyl, and alkoxy.

In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.,* U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913

(1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.,* U.S. Patent 5,539,083), antibody libraries (*see, e.g.,* Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see, e.g.,* Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.,* benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like). Additional examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Some exemplary libraries are used to generate variants from a particular lead compound. One method includes generating a combinatorial library in which one or more functional groups of the lead compound are varied, e.g., by derivatization. Thus, the combinatorial library can include a class of compounds which have a common structural feature (e.g., framework).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.,* 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.,* ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

The test compounds of the present invention can also be obtained from: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g., Zuckermann, R.N. *et al.* (1994) *J. Med. Chem.* 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological libraries include libraries of nucleic acids and libraries of proteins. Some nucleic acid libraries encode a diverse set of proteins (e.g., natural and artificial proteins; others provide, for example, functional RNA and DNA molecules such as nucleic acid aptamers or ribozymes. A peptoid library can be made to include structures similar to a peptide library. (See also Lam (1997) *Anticancer Drug Des.* 12:145). A library of proteins may be produced by an expression library or a display library (e.g., a phage display library).

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici (1991) *J. Mol. Biol.* 222:301-310; Ladner *supra.*).

Information from an assay described herein (e.g., a separation-based or coupled-enzyme assay) can be stored on a computer-readable medium, e.g., in a database, e.g., a relational database. The database can related parameters detected by the assay with sample information (e.g., test compound identity, reaction conditions, patient source, enzyme source (e.g., to evaluate mutated proteins for enzymatic activity), etc.).

After using an assay described herein, the test compound can be assayed in vivo or in the presence of a cell, e.g., a cultured cell. A cell-based assay can include evaluating cell proliferation, cell differentiation, and cell apoptosis.

Structural Activity Relationships

It is also possible to use structure-activity relationships (SAR) and structure-based design principles to find compounds that have improved effects on a target protein, e.g., a nicotinamide releasing activity, e.g., a SIRT activity. SARs provide information about the activity of related compounds in at least one relevant assay. Correlations are made between structural features of a compound of interest and an activity. For example, it may be possible by evaluating SARs for a family of compounds that interact with a target protein to identify one or more structural features required for the interaction. A library of compounds can then be produced that vary these features, and then the library is screened. Structure-based design can include determining a structural model of the physical interaction of the compound and its target. The structural model can indicate how an antagonist of the target can be engineered.

Both the SAR and the structure-based design approach can be used to identify a pharmacophore. Pharmacophores are a highly valuable and useful concept in drug discovery and drug-lead optimization. A pharmacophore is defined as a distinct three dimensional (3D) arrangement of chemical groups essential for biological activity. Since a pharmaceutically active molecule must interact with one or more molecular structures within the body of the subject in order to be effective, and the desired functional properties of the molecule are derived from these interactions, each active compound must contain a distinct arrangement of chemical groups which enable this interaction to occur. The chemical groups, commonly termed descriptor centers, can be represented by (a) an atom or group of atoms; (b) pseudo-atoms, for example a center of a ring, or the center of mass of a molecule; (c) vectors, for example atomic pairs, electron lone pair directions, or the normal to a plane. Once formulated a pharmacophore can be used to search a database of chemical compound, e.g., for those having a structure compatible with the pharmacophore. See, for example, U.S. 6,343,257 ; Y. C. Martin, 3D Database Searching in Drug Design, J. Med. Chem. 35, 2145(1992); and A. C. Good and J. S. Mason, Three Dimensional Structure Database Searches, Reviews in Comp. Chem. 7, 67(1996). Database search queries are based not only on chemical property information but also on precise geometric information.

Computer-based approaches can use database searching to find matching templates; Y. C. Martin, Database searching in drug design, J. Medicinal Chemistry, vol. 35, pp 2145-54

(1992), which is herein incorporated by reference. Existing methods for searching 2-D and 3-D databases of compounds are applicable. Lederle of American Cyanamid (Pearl River, N.Y.) has pioneered molecular shape-searching, 3D searching and trend-vectors of databases. Commercial vendors and other research groups also provide searching capabilities (MACSS-3D, Molecular Design Ltd. (San Leandro, Calif.); CAVEAT, Lauri, G. et al., University of California (Berkeley, Calif.); CHEM-X, Chemical Design, Inc. (Mahwah, N.J.)). Software for these searches can be used to analyze databases of potential drug compounds indexed by their significant chemical and geometric structure (e.g., the Standard Drugs File (Derwent Publications Ltd., London, England), the Bielstein database (Bielstein Information, Frankfurt, Germany or Chicago), and the Chemical Registry database (CAS, Columbus, Ohio)).

Once a compound is identified that matches the pharmocophore, it can be tested for activity, e.g., for binding to a component of a target protein and/or for a biological activity, e.g., a nicotinamide-releasing activity.

Purification methods

The assays described herein can be used to evaluate one or more fractions from a purification, e.g., a purification from a natural source (e.g., tissue samples, e.g., human, murine, or bovine tissue) or a recombinant source. Recombinant proteins can be purified from any suitable expression system. The method can be used to identify a peak of activity, e.g., a fraction that contains a nicotinamide-releasing activity, e.g., a histone deacetylase, e.g., a SIRT activity

Proteins may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, affinity purification, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra;* and Sambrook *et al., supra*). In one embodiment, recombinant Proteins can include an affinity tag that can be used for purification, e.g., in combination with other steps. For example, Crute et al. (1998) *J. Biol. Chem.* 273:35347-35354 describe use of a glutathione-S-transferase N-terminal tag to purify recombinant proteins.

Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein. Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purifying proteins from inclusion bodies. *See, e.g.*, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*). If the proteins are soluble or exported to the periplasm, they can be obtained from cell lysates or periplasmic preparations.

Differential Precipitation. Salting-in or out can be used to selectively precipitate a protein. An exemplary salt is ammonium sulfate. Ammonium sulfate precipitates proteins on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration precipitates many of the more hydrophobic proteins. The precipitate is analyzed to determine if the protein of interest is precipitated or in the supernatant. Ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration.

Column chromatography. Proteins can be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech). See, generally, Scopes, *Protein Purification: Principles and Practice* (1982).

In one embodiment, each fraction (or at least a plurality) of fractions is evaluated by a method described herein. The fractions can be evaluated in parallel. The method can be used to identify a peak of activity, e.g., a fraction that contains a nicotinamide-releasing activity, e.g., a histone deacetylase, e.g., a SIRT activity.

EXAMPLES

Example 1. Boronate resin in filter-plate retains NAD but not nicotinamide.

^{14}C-NAD and ^{14}C-nicotinamide were separately mixed with boronate resin in a Multiscreen filter plate. Scintillation counting of the filtrate showed that NAD was retained by the resin whereas nicotinamide flowed through in the filtrate. Comparison with the total counts added (filtration through Multiscreen plate alone) showed that up to 5 mM NAD was completely bound by the resin and that nicotinamide was not retained by the resin (FIG. 9).

Example 2. Use of a microplate filtration assay to assay the SIRT class of enzymes and determine kinetic parameters.

Human SIRT1 deacetylase was assayed as shown in the scheme in FIG. 2. Enzyme was incubated at 37 °C with ^{14}C-NAD and acetylated peptide [HLKSKKGQSTSRHK(K-Ac)LMFK-OH] (SEQ ID NO:2) in Tris-acetate buffer. After 45 minutes the reaction mixture was diluted with ammonium acetate, pH 9, and transferred to a filter plate containing boronate resin. NAD was retained in the resin while unbound nicotinamide flowed through the filter. Enzyme activity was measured by scintillation counting of the nicotinamide-containing filtrate.

The Michaelis constants (K_M) of NAD and the acetylated peptide substrates were determined by measuring enzyme activity in the presence of varied concentrations of each substrate. The results of these assays are depicted in FIG. 10A and FIG. 10B.

Table 2. Additional Exemplary sequences

Sirtuin 3 (silent mating type information regulation 2, homolog) 3; silent mating type information regulation 2, (S.cerevisiae, homolog)-like 3; sirtuin 3, Mus musculus, GenBank®GI:11967963; Acc.: NP_071878.1
MVGAGISTPSGIPDFRSPGSGLYSNLQQYDIPYPEAIFELGFFFHNPKPFFMLAKELYPGHYRP
NVTHYFLRLLHDKELLLRLYTQNIDGLERASGIPASKLVEAHGTFVTATCTVCRRSFPGEDIWA
DVMADRVPRCAVCTGVVKPDIVFFGEQLPARFLLHMADFALADLLLILGTSLEVEPFASLSEAV
QKSVPRLLINRDLVGPFVLSPRRKDVVQLGDVVHGVERLVDLLGWTQELLDLMQRERGKLDG
QDR (SEQ ID NO:3)

Sirtuin 3; sirtuin (silent mating type information regulation 2, S.cerevisiae, homolog) 3; sirtuin type 3; sir2-like 3; silent mating type information regulation 2, S.cerevisiae, homolog 3, Homo sapiens, GenBank® GI:6912660, Acc. NP_036371.1

MAFWGWRAAAALRLWGRVVERVEAGGGVGPFQACGCRLVLGGRDDVSAGLRGSHGARGEP
LDPARPLQRPPRPEVPRAFRRQPRAAAPSFFFSSIKGGRRSISFSVGASSVVGSGGGSSDKGKLS
LQDVAELIRARACQRVVVMVGAGISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFELPFFFHN
PKPFFTLAKELYPGNYKPNVTHYFLRLLHDKGLLLRLYTQNIDGLERVSGIPASKLVEAHGTFAS
ATCTVCQRPFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQRFLLHVVDFPMADLLL
ILGTSLEVEPFASLTEAVRSSVPRLLINRDLVGPLAWHPRSRDVAQLGDVVHGVESLVELLGW
TEEMRDLVQRETGKLDGPDK (SEQ ID NO:4)

Sirtuin type 3, Homo sapiens, GenBank® GI:5225322, Acc: AAD40851.1; AF083108_1

MAFWGWRAAAALRLWGRVVERVEAGGGVGPFQACGCRLVLGGRDDVSAGLRGSHGARGEP
LDPARPLQRPPRPEVPRAFRRQPRAAAPSFFFSSIKGGRRSISFSVGASSVVGSGGGSSDKGKLS
LQDVAELIRARACQRVVVMVGAGISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFELPFFFHN
PKPFFTLAKELYPGNYKPNVTHYFLRLLHDKGLLLRLYTQNIDGLERVSGIPASKLVEAHGTFAS
ATCTVCQRPFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQRFLLHVVDFPMADLLL
ILGTSLEVEPFASLTEAVRSSVPRLLINRDLVGP
LAWHPRSRDVAQLGDVVHGVESLVELLGWTEEMRDLVQRETGKLDGPDK (SEQ ID NO:5)

Sirtuin 4; sirtuin (silent mating type information regulation 2, S. cerevisiae, homolog) 4; sirtuin type 4; silent mating type information regulation 2, S. cerevisiae, homolog 4; sir2-like 4, Homo sapiens, GenBank® GI:6912662, Acc: NP_036372.1

MKMSFALTFRSAKGRWIANPSQPCSKASIGLFVPASPPLDPEKVKELQRFITLSKRLLVMTGAG
ISTESGIPDYRSEKVGLYARTDRRPIQHGDFVRSAPIRQRYWARNFVGWPQFSSHQPNPAH
WALSTWEKLGKLYWLVTQNVDALHTKAGSRRLTELHGCMDRVLCLDCGEQTPRGVLQERFQ
VLNPTWSAEAHGLAPDGDVFLSEEQVRSFQVPTCVQCGGHLKPDVVFFGDTVNPDKVDFVH
KRVKEADSLLVVGSSLQVYSGYRFILTAWEKKLPIAILNIGPTRSDDLACLKLNSRCGELLPLID
PC (SEQ ID NO:6)

Sirtuin type 4, Homo sapiens, GenBank® GI:5225324, Acc: AAD40852.1, AF083109_1

MKMSFALTFRSAKGRWIANPSQPCSKASIGLFVPASPPLDPEKVKELQRFITLSKRLLVMTGAG
ISTESGIPDYRSEKVGLYARTDRRPIQHGDFVRSAPIRQRYWARNFVGWPQFSSHQPNPAH
WALSTWEKLGKLYWLVTQNVDALHTKAGSRRLTELHGCMDRVLCLDCGEQTPRGVLQERFQ
VLNPTWSAEAHGLAPDGDVFLSEEQVRSFQVPTCVQCGGHLKPDVVFFGDTVNPDKVDFVH
KRVKEADSLLVVGSSLQVYSGYRFILTAWEKKLPIAILNIGPTRSDDLACLKLNSRCGELLPLID
PC (SEQ ID NO:7)

Sirtuin type 2, Homo sapiens, GenBank® GI: 24474785, Acc: CAD43717.1

MPLAECPSCRCLSSFRSVDFLRNLFSQTLSLGSQKERLLDELTLEGVARYMQSERCRRVICLVG
AGISTSAGIPDFRSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGQFKPTICHY
FMRLLKDKGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEKI

FSEVTLKCEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKVDLLLVMGTSLQVQPFASLISKAPL
STPRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGWKK
ELEDLVRREHASIDAQSGAGVPNPSTSASPKKSPPPAKDEARTTEREKPQ (SEQ ID NO:8)

Sirtuin 2 (silent mating type information regulation 2, homolog) 2; silent mating type information regulation 2, (S.cerevisiae, homolog)-like; sirtuin 2, Mus musculus, GenBank® GI:11967961, Acc: NP_071877.1
MAEPDPSDPLETQAGKVQEAQDSDSDTEGGATGGEAEMDFLRNLFTQTLGLGSQKERLLDEL
TLEGVTRYMQSERCRKVICLVGAGISTSAGIPDFRSPSTGLYANLEKYHLPYPEAIFEISYFKKH
PEPFFALAKELYPGQFKPTICHYFIRLLKEKGLLLRCYTQNIDTLERVAGLEPQDLVEAHGTFYT
SHCVNTSCRKEYTMGWMKEKIFSEATPRCEQCQSVVKPDIVFFGENLPSRFFSCMQSDFSKV
DLLIIMGTSLQVQPFASLISKAPLATPRLLINKEKTGQTDPFLGMMMGLGGGMDFDSKKAYRD
VAWLGDCDQGCLALADLLGWKKELEDLVRREHANIDAQSGSQAPNPSTTISPGKSPPPAKEA
ARTKEKEEQQ (SEQ ID NO:9)

SIRT2, Rattus norvegicus, GenBank® GI: 14029137, Acc: AAK51133.1
MDFLRNLFSQTLSLGSQKERLLDELTLEGVARYMQSERCRRVICLVGAGISTSAGIPDFRSPST
GLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKGLLLRCYT
QNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEKIFSEVTPKCEDCQSLVK
PDIVFFGESLPARFFSCMQSDFLKVDLLLVMGTSLQVQPFASLISKAPLSTPRLLINKEKAGQSD
PFLGMIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGWKKELEDLVRREHASIDAQ
SGAGVPNPSTSASPKKSPPPAKDEARTTEREK
PQ (SEQ ID NO:10)

Sirtuin 5 isoform 2; sir2-like 5; sirtuin type 5; sirtuin (silent mating type information regulation 2, S.cerevisiae, homolog) 5; silent mating type information regulation 2, S.cerevisiae, homolog 5, Homo sapiens, GenBank® GI: 13787215, Acc:NP_112534.1
MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGVSAES
GVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAGHRAIAECE
TRLGKQGRRVVVITQNIDELHRKAGTKNLLEIHGSLFKTRCTSCGVVAENYKSPICPALSGKGA
PEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWFGENLDPAILEEVDRELAHCDLCLVVGTSS
VVYPAAMFAPQVAARGVPVAEFNTETTP
ATNRFSHLISISSLIIIKN (SEQ ID NO:11)

Sirtuin 7; sir2-related protein type 7; sirtuin type 7; sirtuin (silent mating type information regulation 2, S.cerevisiae, homolog) 7; silent mating type information regulation 2, S.cerevisiae, homolog 7, Homo sapiens, GenBank® GI:7706712, Acc: NP_057622.1
MAAGGLSRSERKAAERVRRLREEQQRERLRQVSRILRKAAAERSAEEGRLLAESADLVTELQG
RSRRREGLKRRQEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIPDYRGPNGV
WTLLQKGRSVSAADLSEAEPTLTHMSITRLHEQKLVQHVVSQNCDGLHLRSGLPRTAISELH
GNMYIEVCTSCVPNREYVRVFDVTERTALHRHQTGRTCHKCGTQLRDTIVHFGERGTLGQPL

NWEAATEAASRADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKLYIVNLQWTPKDDWAALKL
HGKCDDVMRLLMAELGLEIPAYSRWQDPIFSLATPLRAGEEGSHSRKSLCRSREEAPPGDRG
APLSSAPILGGWFGRGCTKRTKRKKVT (SEQ ID NO:12)

GenBank® GI:27717613, Acc:XP_234931.1, similar to sirtuin (silent mating type information regulation 2, S. cerevisiae, homolog) 6 [Homo sapiens] [Rattus norvegicus]
MSVNYAAGLSPYADKGKCGLPEIFDPPEELECKVWELARLMWQSSTVVFHTGAGISTASGIPD
FRGPHGVWTMEERGLAPKFDITFENARPSKTHMALVQLERMGFLSFLVSQNVDGLHVRSGFP
RDKLAELHGNMFVEECPKCKTQYVRDTVVGTMGLKATGRLCTVAKARGLRACRGELRDTILD
WEDALPDRDLTLADEASRTADLSVTLGTSLQIRPSGNLPLATKRRGGRLVIVNLQPTKHVCAS
ALPSAPVSPACPLRRTDCPSLWQDRQADLCIHGYVDEVMCKLMKHLGLEIPTWDGPRVLEKA
LPPLPRPVAPKAEPPVHLNGSYKPKPDSPVPHRPPKRVKTEAAAS (SEQ ID NO:13)

GenBank® GI:27690910, Acc: XP_221204.1 similar to sirtuin 7; sir2-related protein type 7; sirtuin type 7; sirtuin (silent mating type information regulation 2, S.cerevisiae, homolog) 7; silent mating type information regulation 2, S.cerevisiae, homolog 7 [Homo sapiens] [Rattus norvegicus]
MAAGGGLSRSERKAAERVRRLREEQQRERLRQVSRILRKAAAERSAEEGRLLAESEDLVTELQ
GRSRRREGLKRRQEEASRGQRVCDDPEELRRKVRELAGAVRSARHLVVYTGAGISTAASIPDY
RGPNGVWTLLQKGRPVSAADLSEAEPTLTHMSITQLHKHKLGLPRTAISELHGNMYIEVSSAQ
RTQGLGDKQMSLTVPSLPQVCTSCIPNREYVRVFDVTERTALHRHLTGRTCHKCGTQLRDTI
VHFGERGTLGQPLNWEAATEAASKADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKLYIVNLQ
WTPKDDWAALKLHGKCDDVMRLLMDELGLEIPVYNRWQDPIFSLATPLRAGEEGSHSRKSL
CRSREEPPPGDQSAPLASATPILGGWFGRGCAKRAKRKKAA (SEQ ID NO:14)

GenBank® GI:7657575, Acc: NP_036370.2, sirtuin 1; sirtuin (silent mating type information regulation 2, S. cerevisiae, homolog) 1; sirtuin type 1; sir2-like 1; SIR2alpha [Homo sapiens]
MADEAALALQPGGSPSAAGADREAASSPAGEPLRKRPRRDGPGLERSPGEPGGAAPEREVPA
AARGCPGAAAAALWREAEAEAAAAGGEQEAQATAAAGEGDNGPGLQGPSREPPLADNLYDE
DDDDEGEEEEEAAAAAIGYRDNLLFGDEIITNGFHSCESDEEDRASHASSSDWTPRPRIGPYT
FVQQHLMIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPKRKKRKDINTIEDAVKLLQ
ECKKIIVLTGAGVSVSCGIPDFRSRDGIYARLAVDFPDLPDPQAMFDIEYFRKDPRPFFKFAKEI
YPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQRIIQCHGSFATASCLICKYKVDC
EAVRGDIFNQVVPRCPRCPADEPLAIMKPEIVFFGENLPEQFHRAMKYDKDEVDLLIVIGSSLK
VRPVALIPSSIPHEVPQILINREPLPHLHFDVELLGDCDVIINELCHRLGGEYAKLCCNPVKLSEI
TEKPPRTQKELAYLSELPPTPLHVSEDSSSPERTSPPDSSVIVTLLDQAAKSNDDLDVSESKGC
MEEKPQEVQTSRNVESIAEQMENPDLKNVGSSTGEKNERTSVAGTVRKCWPNRVAKEQISR
RLDGNQYLFLPPNRYIFHGAEVYSDSEDDVLSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFY
NGLEDEPDVPERAGGAGFGTDGDDQEAINEAISVKQEVTDMNYPSNKS (SEQ ID NO:15)

GenBank® GI: 6320163, Acc: NP_010242.1| regulator of silencing at HML, HMR, telomeres, and rDNA; Sir2p [Saccharomyces cerevisiae]
MTIPHMKYAVSKTSENKVSNTVSPTQDKDAIRKQPDDIINNDEPSHKKIKVAQPDSLRETNTT
DPLGHTKAALGEVASMELKPTNDMDPLAVSAASVVSMSNDVLKPETPKGPIIISKNPSNGIFYG
PSFTKRESLNARMFLKYYGAHKFLDTYLPEDLNSLYIYYLIKLLGFEVKDQALIGTINSIVHINSQ
ERVQDLGSAISVTNVEDPLAKKQTVRLIKDLQRAINKVLCTRLRLSNFFTIDHFIQKLHTARKIL
VLTGAGVSTSLGIPDFRSSEGFYSKIKHLGLDDPQDVFNYNIFMHDPSVFYNIANMVLPPEKIY
SPLHSFIKMLQMKGKLLRNYTQNIDNLESYAGISTDKLVQCHGSFATATCVTCHWNLPGERIF
NKIRNLELPLCPYCYKKRREYFPEGYNNKVGVAASQGSMSERPPYILNSYGVLKPDITFFGEAL
PNKFHKSIREDILECDLLICIGTSLKVAPVSEIVNMVPSHVPQVLINRDPVKHAEFDLSLLGYCD
DIAAMVAQKCGWTIPHKKWNDLKNKNFKCQEKDKGVYVVTSDEHPKTL (SEQ ID NO:16)

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for evaluating a test compound, the method comprising:

(a) providing a sample comprising:

(i) a nicotinamide-releasing activity; and

(ii) a donor substrate (e.g., NAD), wherein the donor substrate comprises a nicotinamide moiety; and

(iii) a test compound;

(b) maintaining the sample under preselected conditions;

(c) contacting the sample to a matrix that preferentially interacts with the donor substrate relative to nicotinamide;

(d) evaluating (i') components of the contacted sample that do not interact with the matrix, or (ii') components of the contacted sample that do interact with the matrix.

2. The method of claim 1 wherein the evaluating comprises assessing a parameter characteristic of components of the contacted sample that do not interact with the matrix.

3. The method of claim 1a wherein the parameter is a function of nicotinamide concentration.

4. The method of claim 1, where the contacting comprises separating components of the contact sample that interact with the matrix from the matrix, thereby separating the donor substrate from the sample

5. The method of claim 1, further comprising comparing the parameter to a reference value.

6. The method of claim 5, wherein the reference value is a corresponding value for a control sample evaluated by the method.

7. The method of claim 1, wherein the matrix covalently bonds to the donor substrate.

8. The method of claim 1, wherein the donor substrate is labelled.

9. The method of claim 8, wherein the nicotinamide moiety is labelled.

10. The method of claim 9, wherein the label is radioactive.

11. The method of claim 1 or 8, wherein the donor substrate is not radioactive.

12. The method of claim 8, wherein the detecting comprises detecting the label.

13. The method of claim 1 wherein the evaluating comprises performing an enzyme-based assay.

14. The method of claim 1 wherein the evaluating comprises spectroscopic detection.

15. The method of claim 1, wherein the separating does not require high-pressure liquid chromatography.

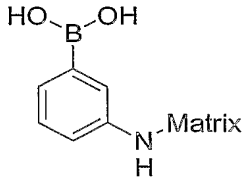
16. The method of claim 1, wherein the separating comprises using one or more of: vacuum, gravity, or centrifugation.

17. The method of claim 1, wherein the separating comprises using a container that comprises a plurality of samples.

18. The method of claim 1, wherein the matrix selectively interacts with compounds having 1,2-diols.

19. The method of claim 18, wherein the matrix includes a boronate group.

20. The method of claim 18, wherein the boronate group is :



21. The method of claim 1 or 18, wherein the donor substrate is NAD, NADH, NADP, or NADPH.

22. The method of claim 1, wherein the nicotinamide-releasing activity is associated with a deacetylase activity (e.g. ability to catalyze deacetylation of an acetylated polypeptide, e.g., an acetylated lysine of a polypeptide).

23. The method of claim 22, wherein the deacetylase activity is associated with a SIRT activity

24. The method of claim 1, wherein the nicotinamide-releasing activity is NAD hydrolase activity.

25. The method of claim 1, wherein a plurality of samples are provided and wherein a plurality of test compounds are evaluated by the method.

26. The method of claim 1, wherein the reaction mixture further comprises an acceptor substrate, wherein the nicotinamide-releasing activity causes the acceptor substrate to become ADP-ribosylated.

27. The method of claim 26, wherein the acceptor substrate is acetylated.

28. A method of evaluating nicotinamide in a sample, the method comprising:

(a) providing a sample;

(b) contacting the sample with a NAD-binding matrix, wherein the matrix does not also bind nicotinamide, under conditions that allow the NAD to bind the matrix;

(c) detecting nicotinamide after the contacting.

29. A kit comprising: a nicotinamide-binding matrix;

NAD;

an acetylated acceptor peptide;

a control enzyme;

and instructions for use in detecting nicotinamide-releasing activity.

30. A method for evaluating a test compound, the method comprising:

(a) providing a sample comprising:

(i) a sample having nicotinamide-releasing activity;

(ii) a donor substrate, wherein the donor substrate comprises a nicotinamide moiety; and

(iii) a test compound;

(b) maintaining the sample under preselected conditions;

(c) contacting the sample with a nicotinamide-modifying activity under conditions that allow the nicotinamide-modifying activity to react with the nicotinamide;

(d) detecting a product of a reaction catalyzed by the nicotinamide-modifying activity.

31. The method of claim 30 wherein the detecting comprises detecting production of a fluorescence or colorimetric product

32. The method of claim 31 wherein the detecting comprises spectroscopy.

33. The method of claim 30, wherein the nicotinamide-modifying activity is nicotinamide deamidase activity.

34. The method of claim 33, wherein the product is ammonia.

35. The method of claim 34, wherein the detecting ammonia comprises:

contacting the reaction mixture with o-phthaldialdehyde (OPA) ; and

detecting optical density in the reaction mixture, thereby detecting levels of ammonia released.

36. The method of claim 30, wherein a plurality of samples are provided and a plurality of test compounds are evaluated.

37. The method of claim 30, wherein the nicotinamide-modifying enzyme is nicotinamide N-methyl transferase, and wherein the modified nicotinamide is detected.

38. The method of claim 37, wherein the contacting step further comprises the steps of:

contacting the sample with acetophenone/KOH and formic acid, and heating the sample.

39. The method of claim 38, wherein the detecting comprises detecting fluorescence (OD).

40. A method of detecting nicotinamide-releasing activity, the method comprising:

(a) providing a sample comprising

(i) nicotinamide-releasing activity, and

(ii) a donor substrate, wherein the donor substrate comprises a nicotinamide moiety;

(b) contacting the reaction mixture with nicotinamide-modifying activity under conditions that allow the enzyme to react with nicotinamide;

(c) detecting one of: a nicotinamide that has been modified by the enzyme, or a byproduct of the nicotinamide-modifying activity.

41. The method of claim 40 wherein the detecting comprises detecting production of a fluorescence or colorimetric product

42. The method of claim 41 wherein the detecting comprises spectroscopy.

43. The method of claim 40, wherein the nicotinamide-modifying activity is nicotinamide deamidase activity.

44. The method of claim 43, wherein the product is ammonia.

45. The method of claim 44, wherein the detecting ammonia comprises:

contacting the reaction mixture with o-phthaldialdehyde (OPA) ; and

detecting optical density in the reaction mixture, thereby detecting levels of ammonia released.

46. The method of claim 40, wherein a plurality of samples are provided and a plurality of test compounds are evaluated.

47. The method of claim 40, wherein the nicotinamide-modifying enzyme is nicotinamide N-methyl transferase, and wherein the modified nicotinamide is detected.

48. The method of claim 47, wherein the contacting step further comprises the steps of:

contacting the sample with acetophenone/KOH and formic acid, and heating the sample.

49. The method of claim 48, wherein the detecting comprises detecting fluorescence (OD).

50. A method of detecting histone deacetylase activity, the method comprising:

providing a sample having histone deacetylase activity;

maintaining the sample under preselected conditions; and

detecting nicotinamide.

51. The method of claim 50, wherein the sample further comprises

a donor substrate, wherein the donor substrate comprises a nicotinamide moiety and wherein the maintaining further comprises

separating the nicotinamide from the donor substrate by binding the sample to a matrix that selectively interacts with the donor substrate but not the nicotinamide, wherein the binding is performed under conditions that allow the unreacted donor substrate to bind the matrix.

52. The method of claim 51, wherein the

sample further comprises a donor substrate, wherein the donor substrate comprises a nicotinamide moiety; and

wherein the maintaining further comprises contacting the sample with a nicotinamide-modifying enzyme under conditions that allow the nicotinamide-modifying enzyme to react with the nicotinamide;

wherein the detecting nicotinamide comprises detecting one of: nicotinamide that has been modified by the enzyme, or a byproduct of the nicotinamide-modifying enzyme.

53. A kit comprising:

a control enzyme having nicotinamide-releasing activity;

a nicotinamide-modifying enzyme;

NAD;

and instructions for detecting one of: modified nicotinamide, or a byproduct of an activity of the nicotinamide-modifying enzyme.

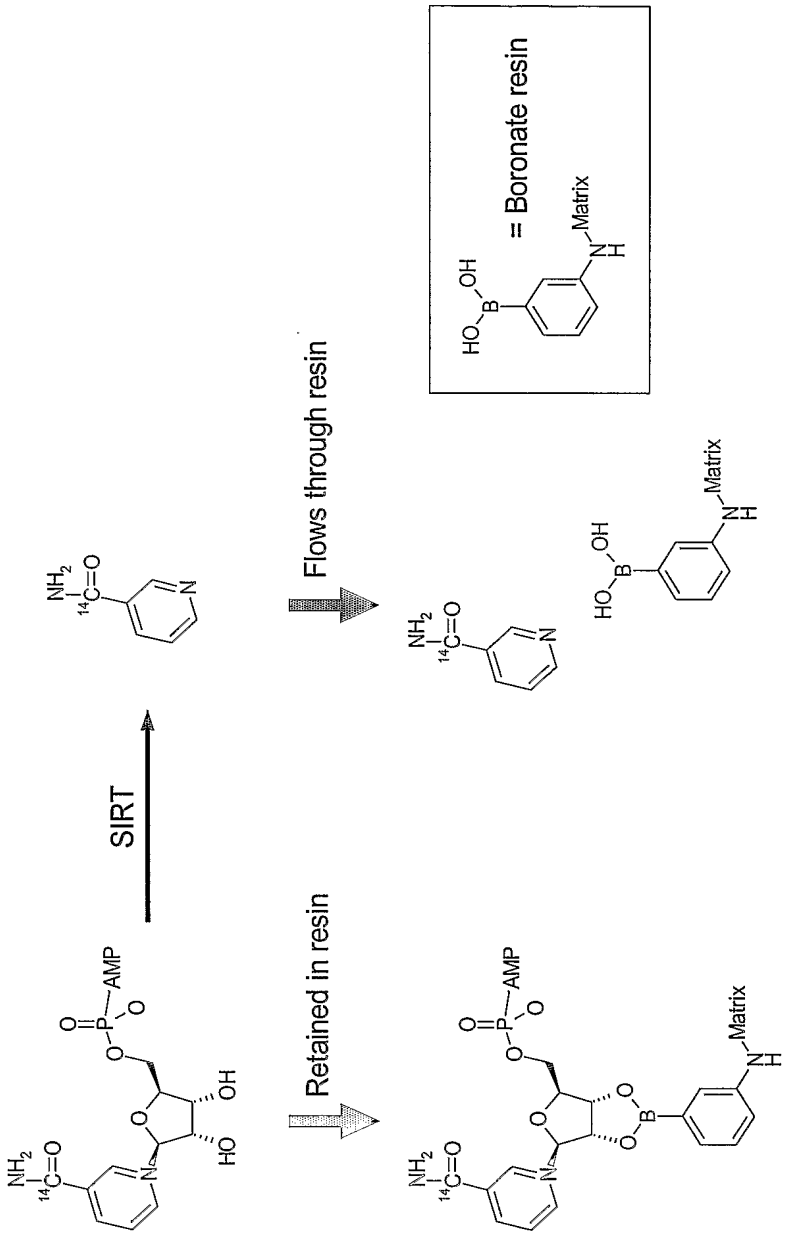

SIRT
Retained in resin
Flows through resin
= Boronate resin
Matrix
AMP

FIG. 1

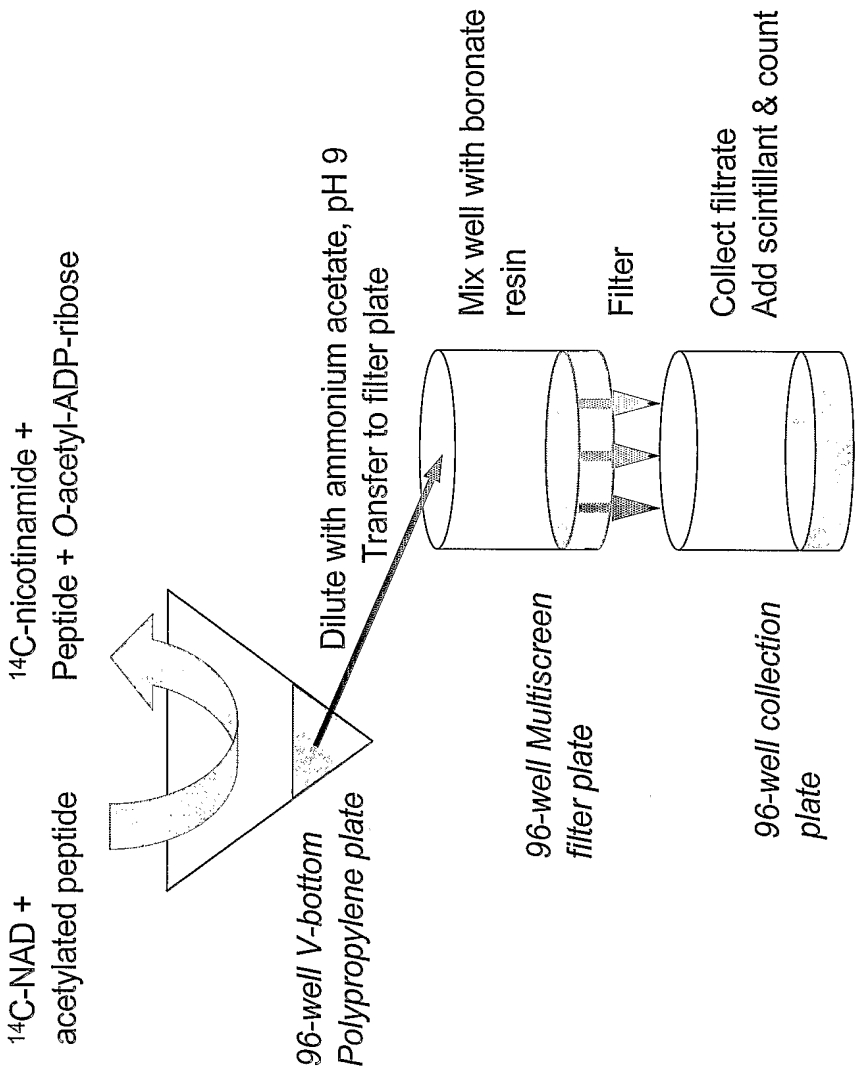

^{14}C-NAD +
acetylated peptide
^{14}C-nicotinamide +
Peptide + O-acetyl-ADP-ribose
96-well V-bottom
Polypropylene plate
Dilute with ammonium acetate, pH 9
Transfer to filter plate
96-well Multiscreen
filter plate
Mix well with boronate
resin
Filter
96-well collection
plate
Collect filtrate
Add scintillant & count

FIG. 2

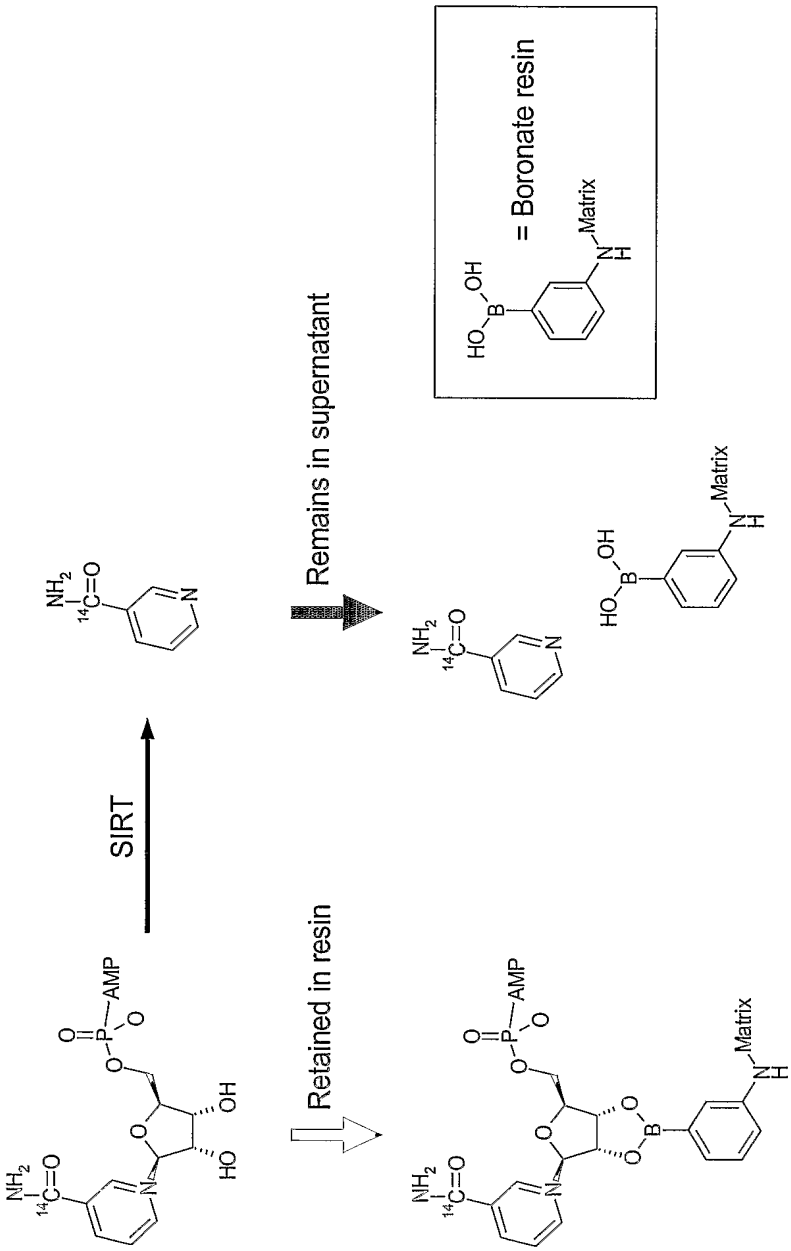

SIRT
Retained in resin
Remains in supernatant
= Boronate resin
Matrix
AMP
OH
HO

FIG. 3

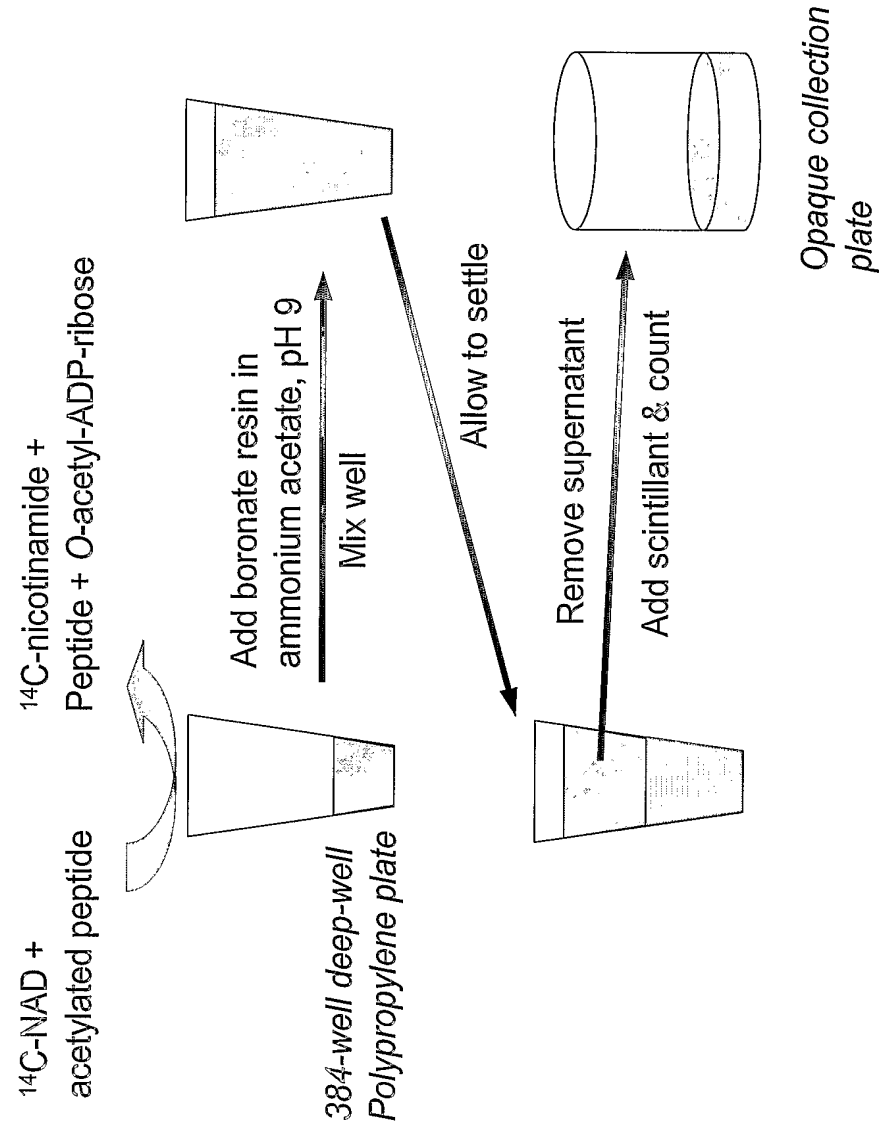

^{14}C-NAD +
acetylated peptide
^{14}C-nicotinamide +
Peptide + O-acetyl-ADP-ribose
384-well deep-well
Polypropylene plate
Add boronate resin in
ammonium acetate, pH 9
Mix well
Allow to settle
Remove supernatant
Add scintillant & count
Opaque collection
plate

FIG. 4

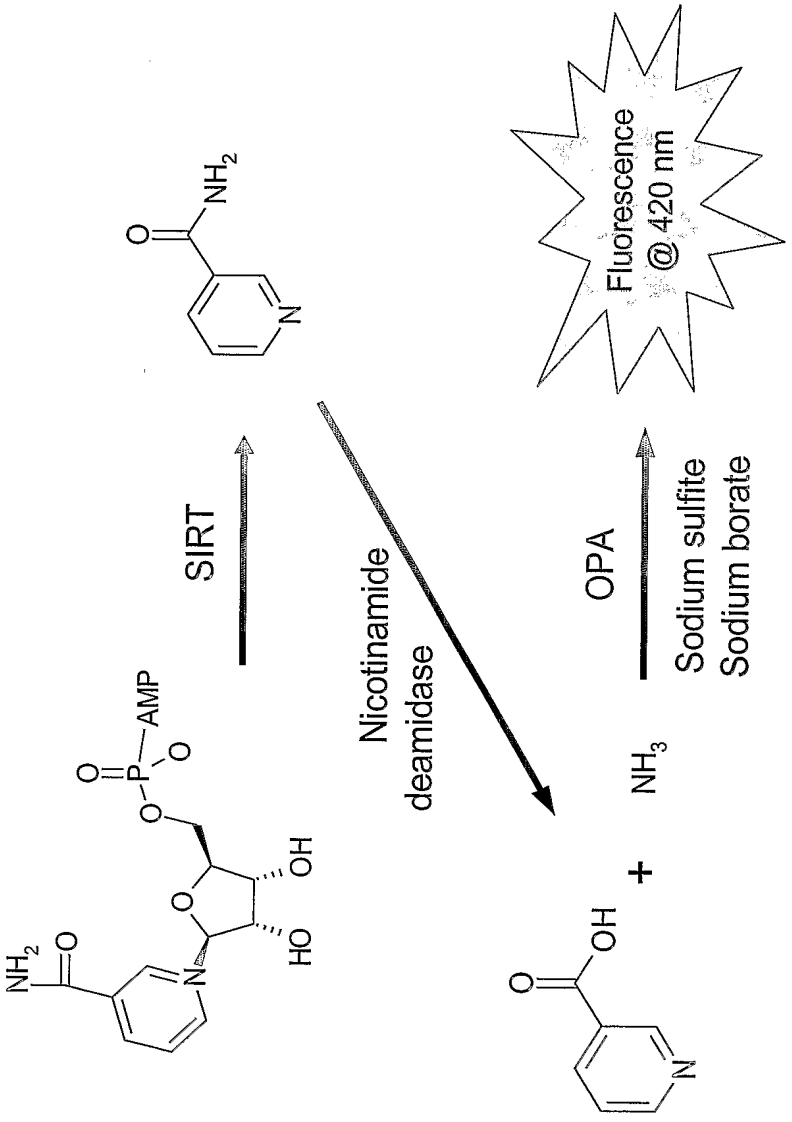

SIRT
Nicotinamide deamidase
OPA
Sodium sulfite
Sodium borate
Fluorescence @ 420 nm
AMP
NH2
NH3
OH
HO

FIG. 5

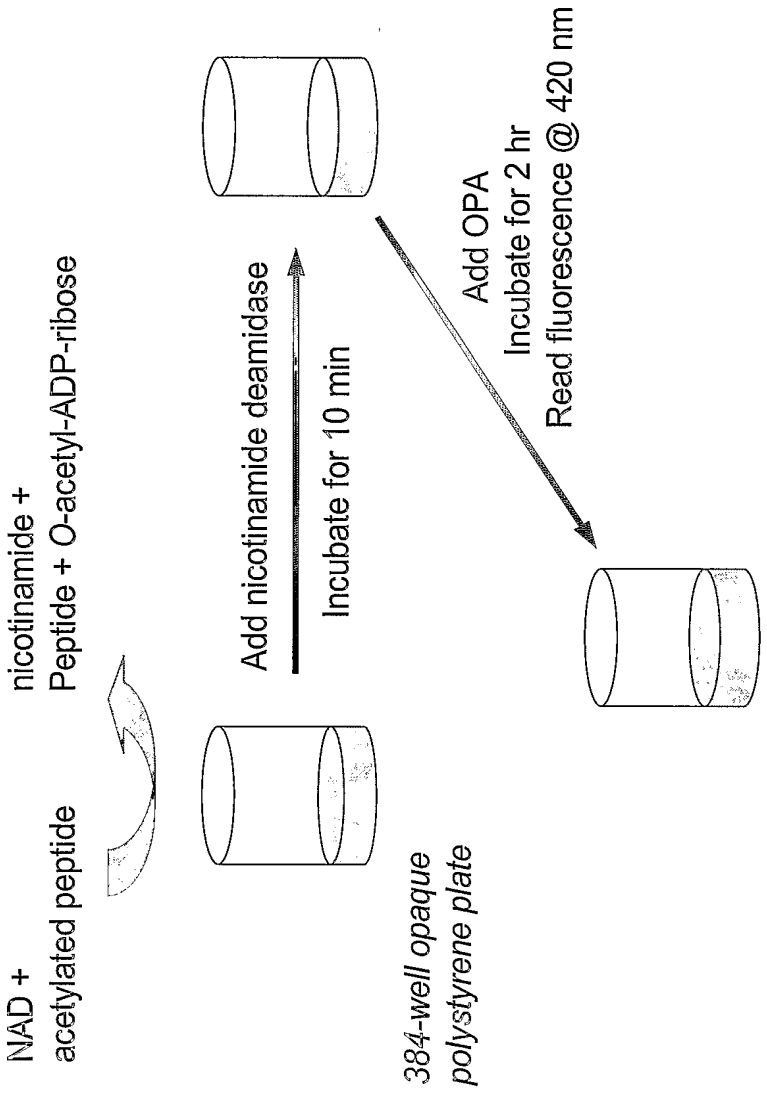

NAD +
acetylated peptide
nicotinamide +
Peptide + O-acetyl-ADP-ribose
384-well opaque
polystyrene plate
Add nicotinamide deamidase
Incubate for 10 min
Add OPA
Incubate for 2 hr
Read fluorescence @ 420 nm

FIG. 6

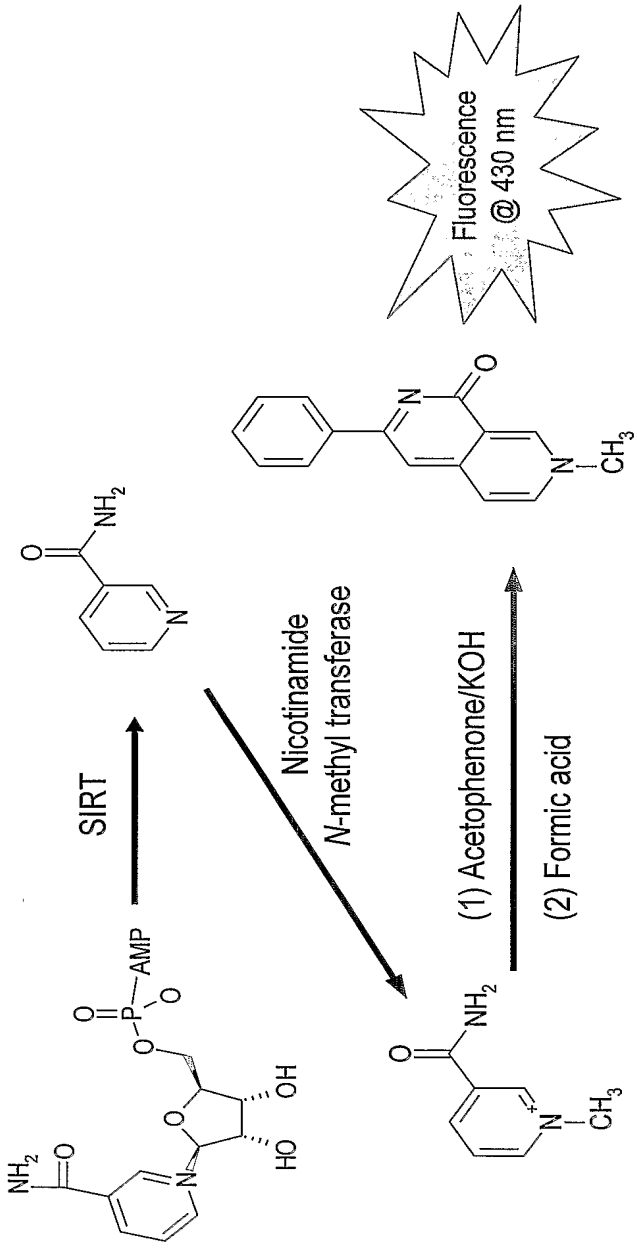

Fluorescence
@ 430 nm
SIRT
Nicotinamide
N-methyl transferase
(1) Acetophenone/KOH
(2) Formic acid
AMP
OH
HO

FIG. 7

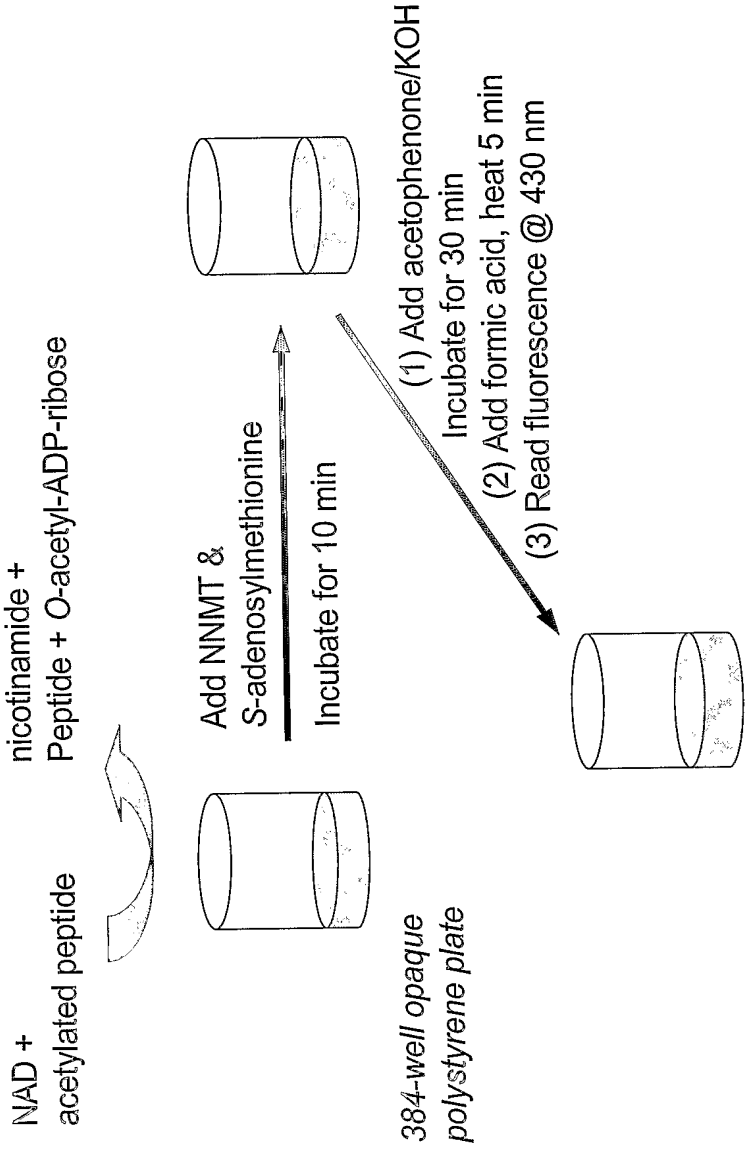

NAD +
acetylated peptide
nicotinamide +
Peptide + O-acetyl-ADP-ribose
Add NNMT &
S-adenosylmethionine
Incubate for 10 min
384-well opaque
polystyrene plate
(1) Add acetophenone/KOH
Incubate for 30 min
(2) Add formic acid, heat 5 min
(3) Read fluorescence @ 430 nm

FIG. 8

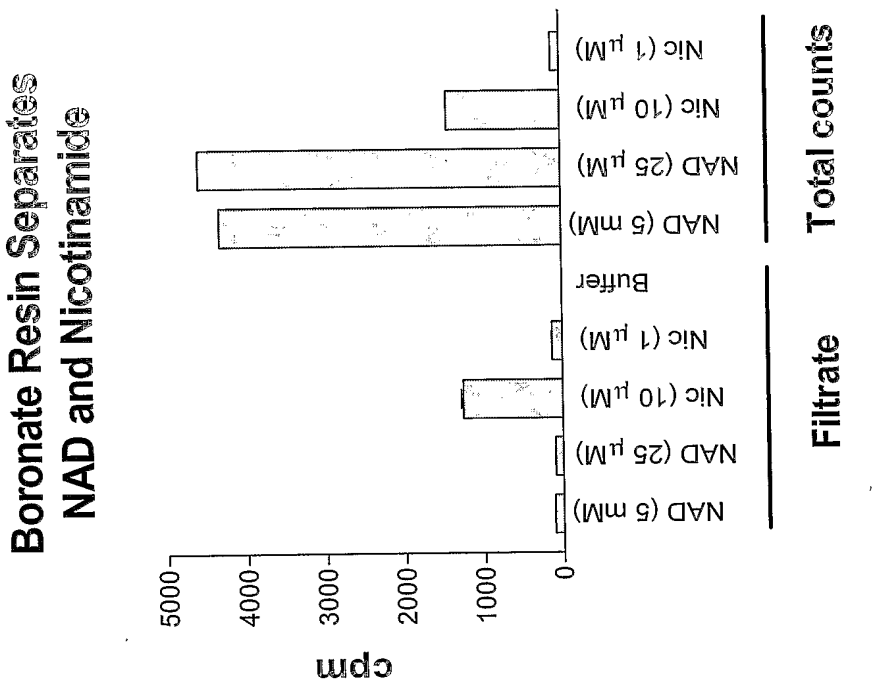

Boronate Resin Separates
NAD and Nicotinamide
cpm
5000
4000
3000
2000
1000
0
NAD (5 mM)
NAD (25 μM)
Nic (10 μM)
Nic (1 μM)
Buffer
NAD (5 mM)
NAD (25 μM)
Nic (10 μM)
Nic (1 μM)
Filtrate
Total counts

FIG. 9

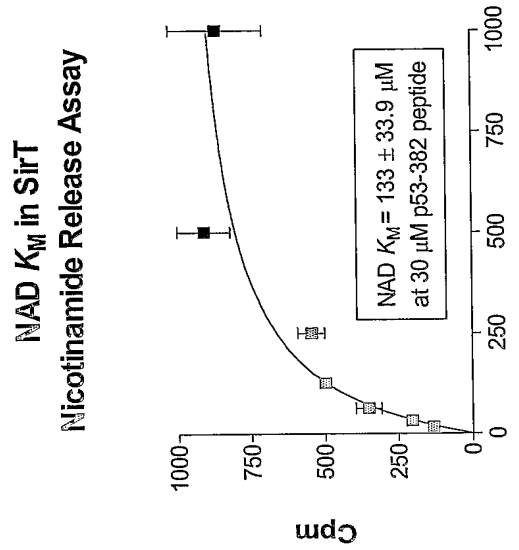


FIG. 10B

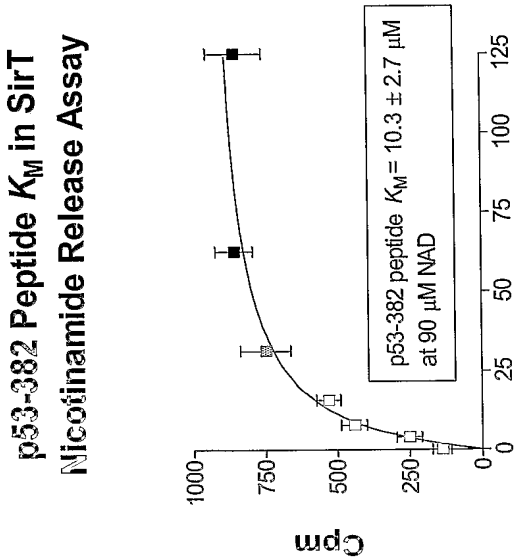


FIG. 10A